REMARKS

Entry of the foregoing, reexamination and further and favorable reconsideration of the subject application in light of the following remarks, pursuant to and consistent with 37 C.F.R. § 1.116, are respectfully requested.

I. CLAIM STATUS & AMENDMENTS

A. Claim Status

According to the Office Action Summary, claims 1-37 were pending in this application when last examined. Claims 11, 12, 17-22, and 27-37 have been examined on the merits. Claims 1-10, 13-16, and 23-26 have been withdrawn from consideration.

The present amendment hereby cancels claims 12, 18, 21 and 22 without prejudice or disclaimer to the subject matter canceled therein. Applicants reserve the right to file a continuation or divisional application on any canceled subject matter. The present amendment also amends claims 11, 17, 21, 27, 34, and 35.

Upon entry of the present amendment, claims 1-11, 13-17, 19, 20, and 23-37 will be pending in this application.

B. Amendments to the Specification & Claims

By the foregoing amendment, the Specification has been amended to make reference to the depository information. Furthermore, claims 12, 18, 21 and 22 have been canceled without prejudice or disclaimer and claims 11, 17, 21, 27, 34, and 35 have been amended.

The Examiner has indicated that the clean and marked up version of claim 11 in the Amendment and Reply dated May 29, 2002 are not the same and that the clean version is being examined. The Examiner has further indicated that claim 17 in the May 29, 2002

Amendment and Reply contains a bracket indicative of a deletion. The amendments made to claims 11 and 17 in the Marked-up copy of the claims attached to the May 29, 2002 Amendment and Reply were inadvertently not transcribed to the clean copy of the claims at the beginning of that document. The present amendment hereby corrects these inadvertent errors. Claims 11 and 27 have been amended to correct inadvertent typographical errors. Claims 11, 17, 21, and 27 have been amended to include the terminology " wherein the percentage of eicosapentaenoic acid to the total fatty acids in said lipid is 0.5% by weight or less." Support for these amendments can be found, at least, in claims 12, 18, and 22. Claims 34 and 35 have been amended to substitute "genus" for subgenus. Support for these amendments can be found in the Specification, at least, at page 6, lines 5-11.

No new matter has been added by any of the foregoing amendments to the Specification or the claims.

II. REJECTION UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 11, 12, 17-22, and 27-37 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. See July 25, 2002 Official Action at pages 2-3. For at least all of the reasons set forth below, Applicants respectfully traverse this rejection.

In particular, the Examiner has indicated that claims 11 and 21, and claims 12 and 22 respectively are substantial duplicates. For the sole purpose of expediting prosecution and not for acquiescing to the Examiner's rejection, the present amendment hereby cancels

claims 21 and 22 without prejudice or disclaimer thereto. Accordingly, these amendments render the rejection moot.

Claim 21 has been indicated as being confusing since the antecedent basis for "fat" in the recitation "of arachidonic acid in fat" is purportedly not clear. For the sole purpose of expediting prosecution and not for acquiescing to the Examiner's rejection, the present amendment hereby cancels claims 21 without prejudice or disclaimer thereto. Accordingly, this amendment renders the rejection moot.

The Examiner further believes that claims 34-37 are confusing because the nature of the taxonomic group "subgenus *Mortierella*" is unclear. For the sole purpose of expediting prosecution and not to acquiesce to the rejection, Applicants have amended the claims to refer to the taxonomic group as the "genus." Accordingly, this amendment renders the rejection moot.

According to the Examiner, claims 11-12, 17-22, and 27-33 are allegedly confusing for not specifying whether the weight percent is dry or wet weight. Applicants respectively traverse this rejection.

The concept of "dry or wet weight percentage" is inapplicable to the claim as written. First, Applicants note that the claim reads on "An isolated lipid containing arachidonic acid wherein the arachidonic acid content to the total fatty acids in the lipid is 50% by weight or more . . . ". Clearly, one skilled in the art would be able to easily ascertain when the arachidonic acid content in the total fatty acids in the lipid is 50% or more by weight regardless as to whether it is a dry weight percentage or a wet weight percentage. Either way, it is still 50 % by weight or more.

Thus, in this regard, the concept of "wet percent or dry percent" weight is not applicable here. To further illustrate this, Applicants submit that the wet percentage is obtained according to the method described in the attached "Official Methods and Recommended Practices of the American Oil Chemists' Society 4th Edition." See the attached copy. In particular, the lipid to be analyzed is hydrolyzed and methyl-esterified so as to convert fatty acids in the lipid to corresponding fatty acid methyl esters. An amount of each fatty acid methyl ester is then determined by gas chromatography, and a percentage of a specific fatty acid methyl ester, such as arachidonic acid methyl ester or eicosapentaenoic acid methyl ester per the total amount of all fatty acid methyl esters is then calculated. Therefore, the concept of "wet percent or dry percent" weight is not applicable.

Second, the rejection fails to set forth an adequate basis as to why one of skill in the art would not be able to ascertain when the arachidonic acid content in the total fatty acids in the lipid is 50% or more by weight regardless as to whether it is a dry weight percentage or a wet weight percentage.

In view of the above, Applicants respectfully request the withdrawal of the Examiner's rejection of the claims under 35 U.S.C. § 112, second paragraph.

IV. REJECTION UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

Claims 36 and 37 have been rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was purportedly not described in the Specification in such

a way as to enable one skilled in the art to make and/or use the invention. See July 25, 2002 Official Action, page 3.

According to the Examiner, claims 36 and 37 allegedly employ a novel strain of *M. alpina* to obtain a specific product. As such, the Examiner has indicated that referral to an appropriate deposit under the Budapest Treaty is required.

Applicants note that the mutant strain, *Mortierella alpina* SAM2153, has been deposited and accepted for deposit under the terms of the Budapest Treaty. Specifically, *Mortierella alpina* SAM2153 was deposited as a national deposition in Japan on August 5, 1996 and was given the deposit Accession No. FERM P-15767. On July 26, 1999, the International Depository Authority accepted a request for transfer to a deposit under the Budapest Treaty and was thus given the deposit Accession No. BP-6794.

Applicants are in the process of preparing a signed declaration pursuant to 37 C.F.R. § 1.808 regarding the restrictions on the availability of the deposited biological material. Applicants will submit this declaration as soon as possible.

Applicants have further amended the Specification at page 12 such that the Specification identifies the organism by the deposit (accession) number, and includes the date of deposit, the name and address of the depository. As further proof that the deposit has been made, a copy of the receipt of deposit, and an English language translation thereof, is attached herewith.

Since the biological material was deposited and accepted under the terms of the Budapest Treaty, Applicants respectfully request withdrawal of this rejection under 35 U.S.C. § 112, first paragraph.

V. REJECTION UNDER 35 U.S.C. § 102 AND § 103(a)

Claims 11, 12, 17-22 and 27-37 have been rejected under 35 U.S.C. § 102(b) as being anticipated by, or in the alternative, under 35 U.S.C. § 103(a) as being obvious over Totani *et al.*, IND. APPL. SINGLE CELL OILS, Ed. Kyle et al., American Oil Chemists Society, Champaign, Illinois, pp. 52-60 (1992) ("Totani"). See Official Action, pages 4-5 and 4.

Claims 11-12 and 17-22 have also been rejected under 35 U.S.C. § 102(b) as being anticipated by or, in the alternative, under 25 U.S.C. § 103(a) as being obvious over Li *et al.*, THE CANADIAN JOURNAL OF CHEMICAL ENGINEERING, 73:135-139 (1995) ("Li"). See Official Action, pages 5-7.

For at least all of the reasons set forth below, Applicants respectfully traverse these rejections. Given that the Examiner has used the same arguments for both rejections, Applicants address both rejections together below.

The cited references fail to either anticipate or render obvious the claimed invention because they fail to teach or suggest each and every limitation of the claimed invention.

To anticipate a claim, a single prior art reference must teach, either expressly or inherently, each and every element of the claimed invention. See M.P.E.P. § 2131; Verdegaal Bros. v. Union Oil Co. of California, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987); Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379, 231 U.S.P.Q. 81, 90 (Fed. Cir. 1986).

In addition, to establish a *prima facie* case of obviousness, three criteria must be met. First, there must be some suggestion or motivation in the reference to combine either

the references themselves or to modify or combine the reference teachings. See M.P.E.P. § 2143; In re Vaeck, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991). This element requires that the Examiner must show "some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art that would lead that individual to combine the relevant teachings of the references." In re Fine, 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596, 1598 (Fed. Cir. 1988). In other words, the Examiner must provide a logical reason as disclosed in the prior art at the time of the invention for combining the references along the lines of the invention; otherwise the use of such teachings as evidence of non-obviousness will entail impermissible hindsight. Ex parte Stauber and Eberle, 208 U.S.P.Q. 945, 946 (Bd. App. 1980).

Second, the prior art must provide a reasonable expectation of success. See M.P.E.P. § 2143.02; Vaeck, 947 F.2d at 488, 20 U.S.P.Q.2d at 1438; In re Merck & Co., Inc., 800 F.2d 1091, 231 U.S.P.Q. 375 (Fed. Cir. 1986).

Third, the prior art references must teach or suggest each and every element of the claimed invention. See M.P.E.P. § 2143.03; In re Royka, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974); In re Wilson, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494, 496 (C.C.P.A. 1970).

In the instant case, the cited references fail to either anticipate or render obvious the claimed invention because they fail to teach or suggest each and every limitation of the claimed invention. In particular, the claimed lipid composition is characterized by a combination of two properties: (1) the content of arachidonic acid per total fatty acids in the lipid is 72% or 50% by weight or more, **and** (2) the content of eicosapentaenoic acid

per total fatty acids in the lipid is 0.5% or less. In other words, the lipid of the present invention is characterized by a combination of a high content of arachidonic acid **and** a low content of eicosapentaenoic acid.

Applicants were the first to develop this unique lipid by culturing a novel mutant microorganism in which ω3 desaturase activity has been decreased or is lacking, and which has been obtained by mutating a microorganism capable of producing arachidonic acid and belonging to the genus *Mortierella*.

Neither of the cited references describe a lipid simultaneously having both of these properties. Similarly, neither reference suggests that such a lipid can be produced. In fact, neither reference discloses a lipid having a low content of eicosapentaenoic acid. Thus, for this reason alone the rejection should be withdrawn.

Nonetheless, the Examiner indicated that Applicant's arguments in the Amendment and Reply dated May 29, 2002 are unpersuasive because the arguments were directed to the process steps of using a mutant having decreased ω3 desaturase, whereas the claims are directed to a product-by-process. In this regard, the Examiner argues that the patentability of a product does not depend upon its method of production.

However, as discussed above, the lipid of the present invention contains both a high ratio of arachidonic acid **and** a low content of eicosapentaenoic acid. Applicant's arguments in the previous Amendment and Reply did discuss the failure of the methods of the prior art to describe or suggest the novel means used in the present invention. These arguments also pointed out that the prior art references do not produce the claimed lipid of the present invention. In particular, the prior art references fail to describe or suggest the

lipid of the present invention that contains both a high ratio of arachidonic acid **and** a low content of eicosapentaenoic acid.

Thus, for at least all of the reasons set forth above, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 102(b), or in the alternative, under 35 U.S.C. § 103(a).

CONCLUSION

In the event that there are any questions relating to this Amendment and Reply, or the application in general, the Examiner is invited to telephone the undersigned concerning such questions so that prosecution of this application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: _____
Jay F. Williams
Registration No. 48,036

P.O. Box 1404
Alexandria, Virginia 22313-1404
(703) 836-6620

Date: January 27, 2003

Attachment to Amendment and Reply dated January 27, 2003

Marked-up Copy of Amended Claims 11, 17, 21, 27, 34, and 35

([bracketed] items deleted; underlined items added)

11. (Twice Amended) An isolated arachidonic acid-containing microbial lipid containing 72% by weight or more of arachidonic acid to the total fatty acids in said lipid, wherein said lipid is obtainable by extracting microbial cells with an organic solvent, and wherein the percentage of eicosapentaenoic acid to the total fatty acids in said lipid is 0.5% by weight or less.

17. (Twice Amended) An isolated lipid containing arachidonic acid wherein the arachidonic acid content in the total fatty acids in the lipid is 50% by weight or more, wherein the percentage of eicosapentaenoic acid to the total fatty acids in the lipid is 0.5% by weight or less, said lipid being obtained by culturing a microorganism [according to any one of claims 13 to 16] in which ω3 desaturase activity has been decreased or is lacking, wherein said lipid is obtainable by extracting microbial cells with an organic solvent, and further wherein said microorganism is obtained by the mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus *Mortierella*, the genus *Conidiobolus*, the genus *Pythium*, the genus *Phytophthora*, the genus [*Penicullium*] *Penicillium*, the genus *Cladosporium*, the genus *Mucor*, the genus *Fusarium*, the genus *Aspergillus*, the genus *Rhodotorula*, the genus *Entomophthora*, the genus *Echinosporangium* or the genus *Saprolegnia*.

Attachment to Amendment and Reply dated January 27, 2003

Marked-up Copy of Amended Claims 11, 17, 21, 27, 34, and 35

([bracketed] items deleted; underlined items added)

21. (Amended) An arachidonic acid-containing microbial lipid containing 72% by weight or more of arachidonic acid in fat per total fatty acids in said fat, wherein the percentage of eicosapentaenoic acid to the total fatty acids in said lipid is 0.5% by weight or less.

27. (Amended) An isolated arachidonic acid-containing microbial lipid, wherein the [arachidonic] arachidonic acid content per the total fatty acid in the lipid is 50% by weight or more, wherein the percentage of eicosapentaenoic acid to the total fatty acids in the lipid is 0.5% by weight or less, and wherein the lipid is obtainable by culturing a microorganism in which ω3 desaturase activity has been decreased or is lacking at a temperature lower than the optimum growth temperature from the start of culturing or after culturing at the optimum growth temperature, said microorganism being obtained by mutagenesis of a microorganism capable of producing arachidonic acid and belonging to the genus *Mortierella*, the genus *Conidiobolus*, the genus *Pythium*, the genus *Phytophthora*, the genus [*Penicullium*] *Penicillium*, the genus *Cladosporium*, the genus *Mucor*, the genus *Fusarium*, the genus *Aspergillus*, the genus *Rhodotorula*, the genus *Entomophthora*, the genus *Echinosporangium* or the genus *Saprolegnia*; and then

recovering said arachidonic acid-containing microbial lipid from the culture.

Attachment to Amendment and Reply dated January 27, 2003

Marked-up Copy of Amended Claims 11, 17, 21, 27, 34, and 35

([bracketed] items deleted; underlined items added)

34. (Amended) The lipid according to claim 27, wherein the microorganism belongs to the [subgenus] genus *Mortierella*.

35. (Amended) The lipid according to claim 34, wherein the microorganism belonging to the [subgenus] genus *Mortierella* is *Mortierella alpina*.

Attachment to Amendment and Reply dated January 27, 2003

Marked-up Copy of Amended Specification

([bracketed] items deleted; underlined items added)

Marked-up copy of the amended paragraph at page 12, lines 3-12:

-- As mutant strains of the present invention, for example, [Mortierella alpina] *Mortierella alpina* SAM2153 (Accession No. FERM P-15767) (Accession No. FERM BP-6794) wherein ω3 desaturase activity has been extremely decreased that was induced by the present inventors from [Mortierella alpina] *Mortierella alpina* IFO8568 capable of producing arachidonic acid can be used, but it is not limiting said to strain, and all other mutant strains that exhibit the ratio of an activity of smaller than 1 relative to eicosapentaenoic acid of the parent strain cultured under a low-temperature condition set as 1. The mutant strain, *Mortierella alpina* SAM2153, was deposited as a national deposition in Japan on August 5, 1996 and was given the deposit Accession No. FERM P-15767. On July 26, 1999, the International Depository Authority accepted a request for transfer to a deposit under the Budapest Treaty and was thus given the deposit Accession No. BP-6794. The International Depository Authority is National Institute of Science and Human-Technology Agency of Industrial Science and Technology which is located at 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan. --

国際様式　　INTERNATI　L FORM

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

[特許手続上の微生物の寄託の国際的承認に関するブダペスト条約]

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

下記国際寄託当局によって規則7．1に従い発行される。

issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page.

原寄託についての受託証

寄託者
氏名（名称）　サントリー株式会社
代表取締役　鳥井　信一郎　殿
あて名　〒
大阪府大阪市北区堂島浜２丁目１番４０号

1．微生物の表示

（寄託者が付した識別のための表示）	（受託番号）
Mortierella alpina SAM2153	FERM BP－ 6794

2．科学的性質及び分類学上の位置

１欄の微生物には、次の事項を記載した文書が添付されていた。

■　科学的性質
■　分類学上の位置

3．受領及び受託

本国際寄託当局は、平成　８年　８月　５日（原寄託日）に受領した１欄の微生物を受託する。

4．移管請求の受領

本国際寄託当局は、平成　８年　８月　５日（原寄託日）に１欄の微生物を受領した。
そして、平成１１年　７月２６日に原寄託よりブダペスト条約に基づく寄託への移管請求を受領した。
（平成　８年　８月　５日に寄託された微工研菌寄第Ｐ－　１５７６７　号より移管）

5．国際寄託当局

通商産業省工業技術院生命工学工業技術研究所

名　称：　National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology

所　長　大箸　信一郎
Dr. Shinichiro Ohashi Director-General

あて名：　日本国茨城県つくば市東１丁目１番３号（郵便番号３０５－８５６６）
1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305-8566, JAPAN

平成１１年（１９９９）　７月２６日

DEPOSITOR

Name: SUNTORY LIMITED

Representative: Shinichiro Torii

Address: 1-40, Dojimahama 2-chome,
Kita-ku, Osaka-shi, Osaka

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOTITARY AUTHORITY identified at the bottom of this page.

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPODITOR: Mortierella alpina SAM2153

(Deposition Number)

FERM BP-6794

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

× a scientific description

× a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganisms identified under I above, which was received by it on August 5, 1996 (Date of the original deposit)[1].

IV. RECEIPT OF TRANSFER

This International Depositary Authority accepted the microorganisms identified under I above, on August 5, 1996 (Date of the original deposit), and accepted a request for transfer to a deposition under Budapest treaty from the original deposition, on July 26, 1999. (Transferred from FERM P-15767 deposited on August 5, 1996)

V. INTERNATIONAL DEPOSITARY AUTHORITY

National Institute of Science and Human-Technology

Agency of Industrial Science and Technology

Director General Shinichi Ohashi

1- 3, Higashi 1 chome, Tsukuba-shi,
Ibaraki-ken, 305-8566, Japan

July 26, 1999

沖田様 全6枚 ←フ[redacted]用6 3阿鵜

OFFICIAL METHODS
AND
RECOMMENDED PRACTICES
OF THE
AMERICAN OIL CHEMISTS' SOCIETY
FOURTH EDITION

Explanation of Method Numbers:

Each section of methods applying to a specific type of product is designed by a capitol letter. Small letters are introduced where sections are divided into subsections. The first numeral indicates a specific method or test. When more than one such method or test is available, small letters again represent the subdivision. The two numerals following the dash represent the year of initial adoption. The numerals in parentheses indicate the year of the latest issue of the method. Methods are reissued when they are revised, corrected, reapproved or become official.
The heading of the method designates whether the method is official or a recommended practice. Each recommended practice is designated by a black stripe on the right-hand margin.

Surplus Methods:

Method numbers followed by an asterisk (*) have been placed in surplus status by vote of the Uniform Method Committee and will not be printed in future issues of this manual. They will be listed on the index for reference purposes. Individual copies of these methods can be obtained upon request to the offices of the Society.

AOCS Official Method C 1b-89

Revised 1990, 1991, 1992
Reapproved 1993

Fatty Acid Composition by GLC

Marine Oils

Definition: This method determines the fatty acid composition of marine oils and marine oil esters by capillary column gas-liquid chromatography.

Scope: This method is designed to determine the fatty acid composition of marine oils and marine oil esters in relative (area %) values, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in absolute (mg/g) values using a bonded polyglycol liquid phase in a flexible fused silica capillary column. $C_{23:0}$ fatty acid is used as the internal standard. The method is applicable to the analysis of marine oil or marine oil ethyl or methyl esters, capsules of EPA and DHA and minor naturally occurring polyunsaturated fatty acids (see Notes, 1).

Apparatus

1. Gas chromatograph—with capillary injection system (split mode preferred, operated at a split ratio of 1:50; see Notes, 2) and flame-ionization detector (FID; see Notes, 3), capable of meeting the following minimum requirements (see Notes, 4):
   Injection port, 250 C
   Detector, 270 C
   Oven temperature profile:
   Initial temperature, 170 C
   Initial hold time, 0 min
   Program rate, 1.0 C/min
   Final temperature, 225 C
   Final hold time, 0 min
2. The capillary GLC column should be of flexible fused silica, 25 m or more in length and 0.20–0.35 mm i.d. The liquid phase must be bonded Carbowax-20M or an equivalent polyglycol. Examples of the latter are Supelcowax-10, 30 m × 0.25 mm (or 0.32 mm) with 0.25 µm coating (Supelco, Inc., Bellefonte, PA, USA; catalog no. 2-4079 or no. 2-4080) and Omegawax 320, 30 m × 0.32 mm i.d. with 0.25 µm coating (Supelco, Inc., catalog no. 2-4152). No constraints are placed upon the column supplier; however, the manufacturer's claim for the upper temperature limit of the column should be noted and verified.
3. Carrier gas—hydrogen or helium, 99.99% pure or better, is required.
   Note—An oxygen scrubber is mandatory with Carbowax-20M and similar columns.
4. Any suitable amplifier, recorder, integrator or data processor may be used.
5. Constant-temperature water bath or dry heating block—maintained at 100 C.
6. Screw-cap tubes—16 × 125 mm, with leak-tight Teflon™-lined caps.
7. Vial—screw- or crimp-cap, 12 mL.
8. Volumetric pipets—1 and 2 mL.
9. Pasteur-type pipets.
10. Volumetric flasks—25 and 100 mL.
11. Analytical balance—±0.0001 g.
12. Source of dry nitrogen.

Reagents

1. Sodium hydroxide—reagent grade.
2. Methyl alcohol—reagent grade (see Notes, Caution).
3. BF_3—12% in methanol, 2-mL ampules (Supelco, Inc., catalog no. 3-3020, or equivalent).
4. Isooctane—reagent grade (see Notes, Caution).
5. Sodium chloride—reagent grade.
6. $C_{23:0}$ methyl ester—see Notes, 5.
7. $C_{23:0}$ ethyl ester—see Notes, 5.
8. Solutions—
   (a) Alcoholic sodium hydroxide (NaOH), 0.5 N—dissolve 2.0 g NaOH in methanol and make to 100 mL with methanol.
   (b) Sodium chloride (NaCl)—saturated solution, dissolve 36 g NaCl in 100 mL distilled water.

Procedure

1. Oils—
   (a) Accurately weigh (±0.1 mg) approximately 25 mg of $C_{23:0}$ methyl ester internal standard (IS) into a 25-mL volumetric flask and make to volume with isooctane.
   (b) Pipet 1.0-mL aliquots of IS (see Notes, 6) into culture tubes and evaporate the solvent. Store the tubes in a freezer if they will not be used immediately.
   (c) Accurately weigh (±0.1 mg) approximately 25 mg of marine oil sample (may be from fish oil capsule) into the culture tube containing the IS.
   (d) Add 1.5 mL 0.5 N NaOH. Blanket with nitrogen, cap tightly, mix and heat at 100 C for 5 min.
   (e) Cool, add 2 mL BF_3/methanol reagent, blanket with nitrogen, cap tightly, mix and heat at 100 C for 30 min.
   (f) Cool to 30–40 C, add 1 mL of isooctane, blanket with nitrogen, cap and vortex or shake vigorously for 30 sec while still warm.
   (g) Immediately add 5 mL of saturated NaCl solution, blanket with nitrogen, cap and agitate thoroughly.
   (h) Cool to room temperature. When isooctane layer separates from the aqueous layer, transfer the isooctane layer to a clean glass tube, blanket with nitrogen and cap.
   (i) Extract the methanol/water phase again with an additional 1 mL of isooctane.
   (j) Combine isooctane extracts (see Notes, 7) and concentrate to approximately 1 mL with a stream of dry nitrogen.

(k) Inject (see Notes, 8) 1–2 µL under appropriate gas chromatographic conditions (see Apparatus, 1).

2. Methyl or ethyl esters—
   (a) Accurately weigh (± 0.1 mg) approximately 25 mg of $C_{23:0}$ methyl or ethyl ester (appropriate to the substrate to be analyzed) IS into a 25-mL volumetric flask and make to volume with isooctane.
   (b) Pipet a 1.0-mL aliquot (see Notes, 6) of the IS into a culture tube.
   (c) Evaporate the solvent.
   (d) Accurately weigh (± 0.1 mg) no more than 15 mg of esters into the tube containing the IS, add 1 mL of isooctane, blanket with nitrogen, cap and mix thoroughly.
   (e) Inject 1–2 µL under appropriate gas chromatographic conditions (see Apparatus, 1).

   See Figures 1, 2 and 3 for chromatograms of methyl and ethyl esters of various marine oils; see Charts 1 and 2 for retention times of marine oil methyl esters.

Calculations

1. Area percentages—Calculate the area percent for fatty acids by the formula:

$$\text{Area \% fatty acids} = \frac{100\,(A_x)}{A_t - A_{IS}}$$

Where—

A_x = area counts for fatty acid x
A_t = total area counts for the chromatogram
A_{IS} = area counts of the internal standard

2. Calculation of mg of EPA and DHA per g of sample—
   (a) For marine oils and fish oil capsules (methyl esters)—weight of EPA and DHA (see Notes, 9), expressed as mg of EPA/DHA fatty acid per g of oil:

$$\text{EPA or DHA, mg/g} = \frac{(A_x)(W_{IS})(CF_x)}{(A_{IS})(W_S)(1.04)} \times 1000$$

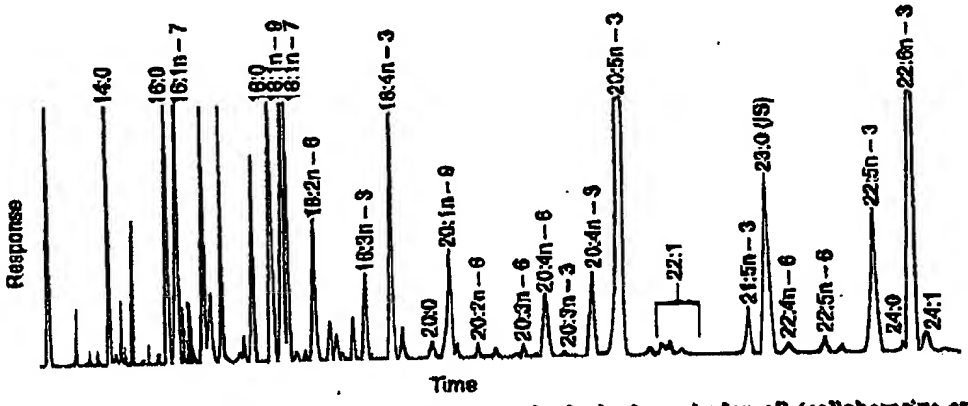

Figure 1. Fatty acid methyl esters of soft-gelatin-encapsulated, steam-deodorized menhaden oil (collaborative study sample 1) analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions noted in the text.

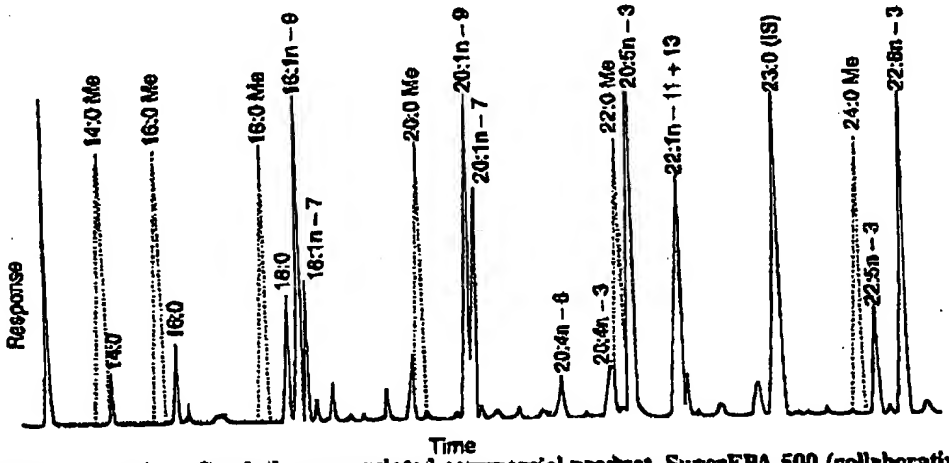

Figure 2. Fatty acid ethyl esters of a soft-gelatin-encapsulated commercial product, SuperEPA 500 (collaborative study sample 2) analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions n ted in the text. The location of added saturated methyl esters is indicated for reference.

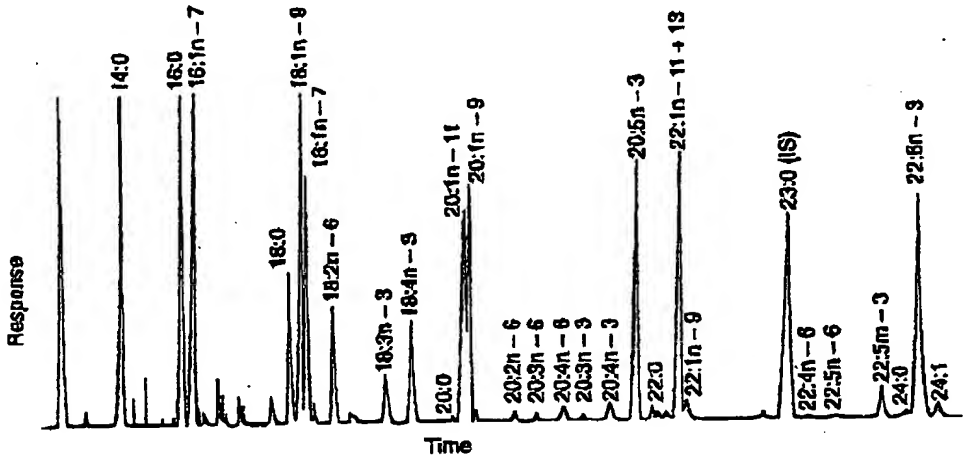

Figure 3. Chromatogram of cod liver oil (collaborative study sample 3) methyl esters analyzed on a flexible fused silica capillary column (30 m × 0.25 mm), coated with Supelcowax-10, under the conditions noted in the text.

(b) For methyl or ethyl esters—weight of EPA and DHA (see Notes, 9), expressed as mg of EPA/DHA fatty acid per g of sample:

$$\text{EPA or DHA, mg/g} = \frac{(A_x)(W_{IS})(CF_x)}{(A_{IS})(W_S)(1.08)} \times 1000$$

Where—

A_x = area counts for EPA (20:5n-3) or DHA (22:6n-3)
A_{IS} = area counts for internal standard
CF_x = theoretical correction factor for EPA or DHA
W_{IS} = weight of internal standard added to the sample, in mg
W_S = sample weight, in mg

3. Detector correction factors—Theoretical detector correction factors relative to $C_{23:0}$ (the internal standard, IS) for $C_{20:5n-3}$ (0.99) and $C_{22:6n-3}$ (0.97) should be applied to the analytical data for optimum accuracy.

Precision

1. Collaborative study statistics, based on results from 19 laboratories that participated in the study, appear in Chart 3.

Notes

Caution

Methanol (methyl alcohol) is flammable and toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume-removal device. Can react vigorously with sodium hydroxide.

Isooctane is highly flammable. An effective fume hood should be used at all times when working with isooctane.

Numbered Notes

1. It has been shown that the alkali-catalyzed methanolysis step for the determination of long-chain marine oil fatty acids produces dimethyl and mixed-alcohol esters from the plasticizer dioctyl [actually di(2-ethylhexyl)] phthalate. An independent boron trifluoride-catalyzed methanolysis step produced a lower level of artifacts, but the official two-step process produced a higher conversion than either catalyst independently. The retention times for the dimethyl, mixed-alcohol and dioctyl phthalates are discussed (References, 1) in relation to methyl esters of common fatty acids on polyglycol wall-coated open-tubular GLC columns.
2. There is usually sufficient sample in marine oils analysis to permit operation in the split mode.
3. The column end may be inserted directly in the detector jet, or there may be a connection at the base of the jet, with or without makeup gas. If the FID requires makeup gas, nitrogen is satisfactory.
4. These are suggested operating conditions for a 30-m column.
5. Authentic standards of saturated fatty acid esters, if needed for peak identification, are widely available. Upon request, the Charleston Laboratory, Southeast Fisheries Center, National Marine Fisheries Service, P. O. Box 12607, Charleston, SC 29412-0607 USA, will provide capsules of collaborative study sample 1 for use in optimizing GLC equipment. $C_{21:5n-3}$ should be baseline separated from $C_{23:0}$ (IS), and $C_{24:0}$ should be baseline separated from $C_{22:6n-3}$ (see Fig. 1).
6. If the peak height of the IS is ≤ one-half that of the EPA or DHA peaks, repeat the analysis using 2.0 mL of IS.
7. Optional—Wash isooctane with 2 mL of water and dry over anhydrous sodium sulfate.
8. Manual injection with a microsyringe is preferred; however, an autoinjection may be used.
9. Please note that this calculation gives results for fatty acids and not for esters of fatty acids. Analyst can confirm detector correction factors with pure reference standards on individual instruments and capillary columns.

Fatty Acid Composition by GLC

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Chart 1. Retention times and area percent of fatty acid methyl esters of steam-deodorized menhaden oil (collaborative study sample 1). See Figure 1.

Fatty acid	Retention time (min)	Relative retention time	Area percent
14:0	4.14	0.335	8.3
16:0	7.13	0.578	18.7
16:1n-7	7.66	0.621	12.1
18:0	12.34	1.00	3.1
18:1n-9	12.97	1.05	11.9
18:1n-7	13.22	1.07	
18:2n-6	14.57	1.18	1.2
18:3n-3	17.08	1.38	0.8
18:4n-3	18.38	1.40	2.9
20:0	20.32	1.65	0.2
20:1n-11	20.91	1.69	1.8
20:1n-9	21.12	1.71	
20:2n-6	23.37	1.89	0.1
20:3n-6	24.66	2.00	0.2
20:3n-3	26.66	2.16	0.2
20:4n-6	25.74	2.09	0.8
20:4n-3	28.07	2.27	1.3
20:5n-3	29.29	2.37	13.8
22:0	30.32	2.46	0.2
22:1n-11+13	31.27	2.53	1.4
22:1n-9	31.63	2.56	
22:4n-6	37.17	3.03	0.2
22:5n-6	38.83	3.14	0.2
22:5n-3	41.11	3.33	2.1
22:6n-3	42.86	3.47	7.9
24:0	42.45	3.44	0.1
24:1n-9	43.54	3.52	0.3

Chart 2. Retention times and area percent of cod liver oil methyl esters (collaborative study sample 3). See Figure 3.

Fatty acid	Retention time (min)	Relative retention time	Area percent
14:0	4.17	0.335	5.7
16:0	7.17	0.576	13.3
16:1n-7	7.71	0.620	8.8
18:0	12.44	1.00	2.3
18:1n-9	13.08	1.05	18.0
18:1n-7	13.32	1.07	
18:2n-6	14.68	1.18	2.0
18:3n-3	17.20	1.38	1.0
18:4n-3	18.50	1.49	2.0
20:0	20.47	1.65	0.2
20:1n-11	21.08	1.69	10.0
20:1n-9	21.28	1.71	
20:2n-6	23.52	1.89	0.3
20:3n-6	24.81	1.99	0.1
20:3n-3	26.86	2.16	0.1
20:4n-6	25.91	2.08	0.4
20:4n-3	28.25	2.27	0.6
20:5n-3	29.43	2.37	7.8
22:0	30.50	2.45	0.3
22:1n-11+13	31.50	2.53	9.0
22:1n-9	31.83	2.56	
22:4n-6	37.70	3.03	0.2
22:5n-6	39.04	3.14	0.1
22:5n-3	41.32	3.32	1.0
22:6n-3	43.77	3.52	7.0
24:0	42.67	3.43	0.2
24:1	43.77	3.52	0.9

Fatty Acid Composition by GLC

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Chart 3

	Samples[a]	1	2	3	4	5	6
$\bar{x}$, %	14:0	8.3	0.4	5.7	7.0	5.4	8.3
	16:0	18.7	0.9	13.3	15.0	7.8	19.0
	18:0	3.1	2.1	2.3	2.9	0.8	3.1
	18:1	11.9	8.7	18.0	14.1	10.2	11.9
	18:4n-3	2.9	1.9	2.0	2.1	4.4	2.9
	20:1	1.8	14.2	10.0	3.2	1.3	1.8
	20:5n-3	13.8	26.4	7.8	17.0	27.5	13.4
	22:1	1.4	10.6	9.0	2.4	1.0	1.3
	22:5n-3	2.0	4.3	1.0	2.1	2.2	2.0
	22:6n-3	7.9	18.7	7.0	12.3	12.5	7.8
RSD_R	14:0	11.7	17.1	13.1	8.3	12.0	11.6
	16:0	7.0	12.2	7.5	6.9	5.7	10.0
	18:0	3.6	7.0	3.1	14.4	5.6	5.7
	18:1	1.9	5.7	2.8	3.0	6.7	3.6
	18:4n-3	2.4	7.6	4.2	6.3	5.5	4.5
	20:1	8.0	3.2	4.5	9.7	18.1	16.2
	20:5n-3	5.8	5.6	5.5	7.4	7.5	9.8
	22:1	17.2	4.7	8.9	10.0	13.4	16.1
	22:5n-3	14.6	8.5	9.7	12.2	8.8	16.2
	22:6n-3	16.1	7.5	9.9	11.7	7.5	15.5
$\bar{x}$, mg/g	20:5n-3	115.3	223.1	64.3	157.5	241.4	117.7
	22:6n-3	66.0	157.9	56.4	105.3	105.4	67.2
RSD_R	20:5n-3	6.0	9.2	7.7	19.8	5.4	7.9
	22:6n-3	4.2	9.0	8.3	12.6	5.2	8.6

RSD_r—Blind duplicate pair (Samples 1 and 6)		$\bar{x}$, %	RSD_r
	14:0	8.3	5.8
	16:0	18.7	2.9
	18:0	3.1	1.8
	18:1	11.9	1.6
	18:4n-3	2.9	1.5
	20:1	1.8	7.3
	20:5n-3	13.8	1.9
	22:1	1.4	7.9
	22:5n-3	2.0	6.7
	22:6n-3	7.9	3.7

$\bar{x}$, mg/g		$\bar{x}$, mg/g	
	20:5n-3	116.5	5.9
	22:6n-3	66.6	5.3

[a]All samples were soft-gelatin encapsulated. 1, Steam-deodorized menhaden oil; 2, commercial ethyl ester preparation; 3, cod liver oil; 4, commercial fish oil; 5, commercial fish oil enriched in EPA and DHA; 6, steam-deodorized menhaden oil (blind duplicate of sample 1).

References

1. *J. Chromatogr. 587*:263 (1991).

Other References

INFORM 1:987 (1990).
J. Am. Oil Chem. Soc. 64:499 (1987).
Ibid. 66:1822 (1989).
General referee report on oils and fats, *J. Assoc. Off. Anal. Chem. 73*:105 (1990).
J. Chromatogr. Biomed. Appl. 533:1 (1990).